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AB Methods for inducing bone formation using ob protein (**leptin**) are disclosed. The methods can be used for treating osteoporosis, repairing fractures, dental defects, resectioning due to oncogenesis and elongation of the growth plate/long bone. In addn., the methods can be used for ex vivo therapy and reinfused into a mammal.

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(57) Abstract

Methods for inducing bone formation using ob protein are disclosed. The methods can be used for treating osteoporosis, repairing fractures, dental defects, resectioning due to oncogenesis and elongation of the growth plate/long bone. In addition, the methods can be used for *ex vivo* therapy and reinfused into a mammal.

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Description

Methods for Inducing Bone Formation

CROSS REFERENCE TO RELATED APPLICATIONS

This application is related to Provisional Application 60/015,647, filed on April 19, 1996. Under 35 U.S.C. § 119(e)(1), this Application claims benefit of said Provisional Application.

BACKGROUND OF THE INVENTION

Bone remodeling is the dynamic process by which tissue mass and skeletal architecture are maintained. The process is a balance between bone resorption and bone formation, with two cell types thought to be the major players. These cells are the osteoblast and osteoclast. Osteoblasts synthesize and deposit matrix to become new bone. The activities of osteoblasts and osteoclasts are regulated by many factors, systemic and local, including growth factors.

While the interaction between local and systemic factors has not been completely elucidated, there is evidence to suggest that some hormones, such as parathyroid hormone (PTH), vitamin D and calcitonin, may be mediated by local factors found in the bone. Some of the growth factors that have been identified in bone include: IGF-I, IGF-II, TGF-β_1, TGF-β_2, bFGF, aFGF, PDGF and the family of bone morphogenic proteins (Baylink et al., J. Bone Mineral Res. 8 (Supp. 2):S565-S572, 1993).

When bone resorption exceeds bone formation, a net loss in bone results, and the propensity for fractures is increased. Decreased bone formation is associated with aging and certain pathological states. Osteoporosis is a disease that afflicts many elderly people, particularly post-menopausal women. In the U.S. alone, there are

approximately 1.5 million fractures annually that are attributed to osteoporosis. The impact of these fractures on the quality of the patient's life is immense. Of people identified with hip fractures due to osteoporosis, 10-20% will die and 50% of sufferers will no longer be able to walk independently (Riggs et al., Bone 17:505S-511S, 1995). Associated costs to the health care system in the U.S. are estimated to be $5-$10 billion annually, excluding long-term care costs.

Currently available treatment for prevention of osteoporosis is limited to salmon calcitonin, hormone replacement therapy (HRT) utilizing estrogen and/or progesterone, and a few bisphosphonate compounds. The worldwide market for salmon calcitonin exceeds $500 million annually. However, patients have been known to develop an immune response to salmon calcitonin, and estrogen therapy has been correlated with a increased risk of uterine and possibly breast cancer. Bisphosphonates have been associated with gastrointestinal problems. Thus, there remains a need for more effective treatments for bone loss.

SUMMARY OF THE INVENTION

It is an object of the present invention to provide methods for stimulating a cell population containing marrow mesenchymal cells comprising: exposing the cell population to an amount of ob protein effective for expansion of osteogenic cells. In one embodiment, the population is enriched for marrow mesenchymal cells. In an embodiment, the hematopoietic cells have been removed. In another embodiment, the osteogenic cells are primarily osteoblasts. In an embodiment, the method further comprises the step of combining the cell population with an anti-resorptive agent. In another embodiment, the method further comprises infusing the cell population into a mammal.

It is a further object of the present invention to provide methods for stimulating a cell population containing marrow mesenchymal cells comprising: exposing the cell population to a biological fluid obtained from an ob protein-treated animal. In another embodiment, the biological fluid is serum. In another embodiment, the biological fluid has been fractionated to enrich for molecules effective for expansion of osteogenic cells.

It is a further object of the present invention to provide methods for stimulating a cell population containing marrow mesenchymal cells comprising: exposing the cell population to culture medium that has been conditioned by growth of endocrine or CNS cells or tissue exposed to ob protein. In another embodiment, the cells are pituitary or hypothalamic cells.

It is a further object of the present invention to provide methods for promoting bone repair or healing in a mammal comprising: administering to the mammal an amount of ob protein effective to provide a clinically significant increase in the bone repair or bone healing rate.

It is a further object of the present invention to provide methods for stimulating bone ingrowth into a prosthetic device or dental implant that has been inserted into a mammal comprising: administering to the mammal amount of ob protein effective to provide a clinically significant increase in bone growth into the device or implant.

It is a further object of the present invention to provide methods for treating bone loss in a mammal comprising: administering to the mammal an amount of ob protein effective to provide a clinically significant increase in bone growth plate width. In another embodiment, the mammal is an adolescent or pre-adolescent.

It is a further object of the present invention to provide methods for stimulating active bone growth in mammal having a fracture in a bone growth plate

comprising: administering to the mammal an amount of ob protein effective to restore a pre-fracture bone length.

It is a further object of the present invention to provide methods for inducing bone formation in mammal having an oncological resection of a bone comprising: administering to the mammal an amount of ob protein effective to induce bone formation at the site of the resection.

DETAILED DESCRIPTION OF THE INVENTION

Prior to describing the present invention in detail, it may be helpful to define certain terms used herein:

ob/ob mice: Inbred mice that are homozygous for an inactivating mutation at the *ob* (obese) locus. *ob/ob* mice are hyperphagic and hypometabolic, and are believed to be deficient in production of circulating satiety factor.

ob: As used herein, *ob* or ob denotes nucleic acid. This designation is distinct from the rodent mutant phenotype designations defined above (i.e., *ob/ob* mice), which are used in the format "*/*", but not in the singular "*".

ob: As used herein, ob denotes protein. Ob protein is also known as "leptin".

stem cell: As used herein, the term "stem cell" refers to a pluripotent cell that has the potential to differentiate into multiple cell types and that is not limited to a fixed number of mitotic divisions.

precursor cell: As used herein, the term "precursor cell" refers to a cell that is committed to a differentiation pathway, but generally does not express markers or function as a mature, fully differentiated cell.

osteogenic cells: As used herein, the term "osteogenic cells" includes osteoblasts and osteoblast precursor cells.

fibroblasts or reticular cells: As used herein, this terms refers to cells of the soft connective tissue network; such cells are found throughout the marrow and spaces in the bone.

adipocytes: As used herein, the term "adipocytes" refers cells distinguished by the presence of their lipid inclusions and expression of specific gene products.

The present invention is based on the discovery that ob protein, when administered to mammals, produces a dramatic increase in osteogenic cells or their bone-forming activity.

The sequence encoding mouse and human ob protein is known (Friedman et al., WO 96/05309). The human and mouse ob DNA and protein sequences are shown in SEQ ID NOS:1, 2, 3 and 4, respectively. Those skilled in the art will recognize that the sequences shown in SEQ ID NOS. 1, 2, 3 and 4 correspond to a single allele of the human and mouse *ob* gene, and that allelic variation is expected to exist. For instance, an allelic variant of the human and mouse *ob* gene does not have a Gln residue at position 49 present.

Ob proteins encoded by allelic variants of the DNA sequence shown in SEQ ID NOS: 1 and 3, including those containing silent mutations and those in which mutations result in amino acid sequence changes, are within the scope of the methods of the present invention, as are ob proteins which are allelic variants of SEQ ID NO: 2 or 4. The present invention contemplates use of DNA molecules encoding ob proteins, wherein the DNA molecules are generally at least 60%, preferably at least 80%, and may be 90-95% or more identical in sequence to SEQ ID NO: 1 or 3 or their allelic variants.

The present invention also contemplates use of isolated proteins that are substantially homologous to the proteins of SEQ ID NO: 2 or 4 and their species homologs. By "isolated" is meant a protein which is found in a

condition other than its native environment, such as apart from blood and animal tissue. In a preferred form, the isolated protein is substantially free of other proteins, particularly other proteins of animal origin. It is preferred to provide the proteins in a highly purified form, i.e., greater than 95% pure, more preferably greater than 99% pure. The term "substantially homologous" is used herein to denote proteins having 50%, preferably at least 80%, sequence identity to the sequence shown in SEQ ID NO: 2 or 4 or their species homologs. Such proteins will more preferably be at least 90% identical, and most preferably 95% or more identical to SEQ ID NO: 2 or 4 or their species homologs. Substantially homologous proteins are characterized as having one or more amino acid substitutions, deletions or additions. These changes are preferably of a minor nature, that is conservative amino acid substitutions that do not significantly affect the folding or activity of the protein; small deletions, typically of one to about 45 amino acids; and small amino- or carboxyl-terminal or internal extensions, such as an amino-terminal methionine residue, a small linker peptide of up to about 20-25 residues, or a small extension that facilitates purification, such as a poly-histidine tract, an antigenic epitope or a binding domain. See, in general Ford et al., Protein Expression and Purification 2: 95-107, 1991, which is incorporated herein by reference. In addition, the ob protein of the present invention (or polypeptide fragments thereof) can be joined to other bioactive molecules, particularly targeting moieties or anti-resorptive agents, to provide multi-functional molecules. Such hybrid ob protein molecules may be formed by chemical conjugation or polynucleotide fusion or a combination thereof, and may further comprise a linker (e.g., a polypeptide linker) between the component proteins or (poly)peptides.

In addition to the ob proteins disclosed above, the methods of the present invention include the use of

fragments of these proteins and isolated polynucleotide molecules encoding the fragments. Of particular interest are ob protein fragments of at least 10 amino acids in length that exhibit bone-forming or osteogenic cell stimulatory activity, and polynucleotide molecules of at least 30 nucleotides in length encoding such polypeptides. Polypeptides of this type are identified by known biological screening and assay methods, and can be produced by proteolytically digesting the intact ob protein or by synthesizing small, overlapping polypeptides or polynucleotides (and expressing the latter). The resultant polypeptide fragments are then tested for bone-forming activity, or for an activity or effect associated with enhanced bone formation. Larger polypeptides up to the size of the entire mature ob protein are also useful herein.

Analysis and modeling of the amino acid sequence shown in SEQ ID NO: 2, using pattern recognition (Cohen et al., Biochem. 25:266-75, 1986) and neural network (Kneller et al., J. Mol. Biol. 214:171-82, 1990) software for the production of secondary structure, predicted that ob protein folds into a four alpha-helix bundle. This helical structure prediction was used as a guide in the generation of a mutiple alignment with other helical cytokines (Buzan, Immunology Today 11: 350-54, 1990; Gribskov et al., PNAS USA 84: 4355-58, 1987). The multiple alignment demonstrated that ob protein has similarity to the helical cytokine structure class. Additional evidence of the possible relatedness of ob protein to the helical cytokine structure class was provided by the two forms of *ob* cDNA (+Gln and -Gln at position 49). These two forms may be caused by slippage at a splice acceptor sequence, indicating the location of a exon-intron boundary (Zhang et al., Nature 372:425-32, 1994). With respect to the multiple alignment, the location of the exon-intron boundary of *ob* was determined to be coincident with an exon-intron boundary in

interleukin-3 and GM-CSF, and is within two residues of exon-intron boundaries in interleukin-2 and interleukin-6.

This prediction is supported with the recent report describing the ob protein receptor (OB-R; Tartaglia et al., Cell 83:1263-71, 1995). OB-R is a membrane-spanning receptor that is closely related to the gp130 signal-transducing component of the IL-6, G-CSF and LIF receptors. The extracellular domain of OB-R shows many similarities to the class I cytokine family.

In a preferred embodiment, a substantially homologous ob protein or ob protein fragment is capable of folding into a four alpha-helix bundle and capable of self-assembly. The amino acid residues that impart the four alpha-helical bundle character of the ob protein molecule are preferably maintained with little or no amino acid substitutions, additions or deletions. However, the remaining amino acid residues may tolerate more extensive amino acid substitutions, additions or deletions with retention of desired biological function.

Essential amino acids in the ob polypeptides useful herein can be identified according to procedures known in the art, such as site-directed mutagenesis or alanine-scanning mutagenesis (Cunningham and Wells, Science 244, 1081-85, 1989). In the latter technique, single alanine mutations are introduced at every residue in the molecule, and the resultant mutant molecules are tested for biological activity (i.e., osteoblast activating activity or bone forming activity) to identify amino acid residues that are critical to the activity of the ob protein polypeptide or fragment. Sites of ligand-receptor interaction can also be determined by analysis of crystal structure, as determined by such techniques as nuclear magnetic resonance, crystallography or photoaffinity labeling. See, for example, de Vos et al., Science 255:306-12, 1992; Smith et al., J. Mol. Biol. 224:899-904, 1992; Wlodaver et al., FEBS Lett. 309:59-64, 1992.

Multiple amino acid substitutions can be made and tested using known methods of mutagenesis and screening, such as those disclosed by Reidhaar-Olson and Sauer (Science 241:53-57, 1988) or Bowie and Sauer (Proc. Natl. Acad. Sci. USA 86:2152-56, 1989). Briefly, these authors disclose methods for simultaneously randomizing two or more positions in a protein, selecting for functional protein, and then sequencing the mutagenized proteins to determine the spectrum of allowable substitutions at each position. These methods allow the rapid determination of the importance of individual amino acid residues in a protein of interest, and can be applied to proteins of unknown structure.

The DNA molecule shown in SEQ ID NO:1 was cloned by polymerase chain reaction (PCR; see Mullis et al., U.S. Patent No. 4,683,195; Mullis, U.S. Patent No. 4,683,202) using a probe designed from the sequence of the mouse *obese* gene (Zhang et al., Nature 372:425-432, 1994) and cDNA from a human adipose tissue cDNA library (obtained from Clontech, Palo Alto, CA). By this method, the DNA molecule shown in SEQ ID NO: 1 was isolated.

Analysis of the amino acid sequence shown in SEQ ID NO:2 indicated that the protein included an amino-terminal signal peptide of 21 amino acid residues. The mature protein thus begins with amino acid residue 22 (Val) of SEQ ID NO:2.

Bone is a tissue and, therefore, contains a composite of heterogeneous cell populations. Some of the cell types found in bone include osteoblasts, osteoclasts, chondrocytes and osteocytes. Examples of other tissues include bone marrow, skin, skeletal and cardiac muscle, pancreas, brain and liver. Tissues usually consist of a mixture of tissue-specific cell types, as well as cell types that are found in many tissues, such as fibroblasts. It is within the bone marrow that many of the earliest progenitor cells are found, and these are generally described as either hematopoietic stem cells or

mesenchymal stem cells. Human hematopoietic stem cells are identified as $CD34^+$, and differentiate to become either myeloid cells (including erythrocytes, megakaryocytes, macrophages and neutrophils) or lymphoid cells (T cells and B cells). Of particular interest herein are cells of the mesenchymal lineage, and includes stem cells and precursor cells for osteoblasts, chondrocytes, adipocytes, fibroblasts and reticular cells (Owen et al., Ciba Fdn. Symp. 136:42-46, 1988). Markers for mesenchymal stem cells have not been well identified (Owen et al., J. of Cell Sci. 87:731-738, 1987), so identification is usually made at the precursor and mature cell stages. As defined herein, mesenchymal cells are histologically distinct from hematopoietic cells. Stromal cells are generally considered the subpopulation of bone marrow cells that are adherent when cultured *in vitro* and are included in the mesenchymal lineage cells.

Differentiation is the process of maturation of cells. It is a progressive and dynamic process, beginning with pluripotent stem cells and ending with terminally differentiated cells that progress no further down the cell lineage pathway. A cell's function, phenotype and growth characteristics are affected by the cell's degree of differentiation. As stem cells or progenitor cells begin the process of differentiation, they generally migrate to selected tissues and/or organs, where they appear to undergo additional differentiation.

Typically, a cell's differentiated state can be identified using a set of differentiation markers that is specific to a particular cell type. Differentiation markers are transiently exhibited at various stages of cell lineage development. Pluripotent stem cells can regenerate without commitment to a lineage, and express a set of differentiation markers that are lost when commitment to a cell lineage is made. Precursor cells express a set of differentiation markers that may or may not continue to be expressed as the cells progress down

the cell lineage pathway toward maturation. Differentiation markers that are expressed exclusively by mature cells are usually associated with functional properties, such as cell products, enzymes to produce cell products and receptors. Mature osteoclasts express a set of differentiation markers that is selected from the group consisting of tartrate-resistant acid phosphatase (TRAP) and calcitonin receptor (Suda et al., Endocrine Rev. 13:66-80, 1992). Identification of osteoblasts is primarily phenotypic, and includes expression of alkaline phosphatase (Manduca et al., J. Bone Min. Res. 8:281, 1993); type 1 collagen synthesis (Kurihara et al., Endocrinol. 118(3):940-947, 1986); production of osteocalcin (Rodan et al., Crit. Rev. Eucar. Gene Expr. 1:85-98, 1991)); and responsiveness to parathyroid hormone (Aubin et al., J. Cell Biol. 92:452-461, 1982).

Other markers of differentiation for specific cell types include: cardiac myosin isozyme expression, a cardiac specific pattern of creatine kinase isozyme expression (Yaffe et al., Develop. Biol. 15:33-50, 1967 and Richler et al., Develop. Biol. 23:1-22, 1970) and insulin and insulin-like growth factor receptors I (Wolleben et al., Am. J. Physiol. 252:E673-E678, 1987) for cardiac muscles cells; myosin isozyme expression and a muscle-specific pattern of creatine kinase isozyme expression (I and II) (Yaffe et al., Develop. Biol. 11:300-317, 1965; Yaffe et al,. Develop. Biol. 15:33-50, 1967; Richler et al., Develop. Biol. 23:1-22, 1970) for skeletal muscle cells; aggrecan (Doege et al., J. Biol. Chem. 266:894-902, 1991) and collagen Type IIB (Sandell et al., J. Cell Biol. 114:1307-1319, 1991) for chondrocytes; mpl receptor (Souyri et al., Cell 63: 1137-1147, 1990) and acetylcholine (Ravid et al., J. Cell. Biol. 123: 1545-1553, 1993) for megakaryocytes; insulin (Powers et al., Diabetes 39: 406-414, 1990) for pancreatic b-cells; glucagon and glucagon-like polypeptide (Lacy et al., Diabetes 16:35, 1967; Gotoh et al., Transplantation

40:437-438, 1985; Hamaguchi et al., Diabetes 40:842-849, 1991) for pancreatic a-cells; somatostatin (Williams et al. Somatostatin and Pancreatic Polypeptide in International Textbook of Diabetes Mellitus, Alberti et al., eds., 1992) for pancreatic d-cells; pOb24, lipoprotien lipase, AP2, PPARγ (Butterwith, Pharmac. Ther. 61:399-411, 1994), triglyceride and perilipin (Greenberg et al., J. Biol. Chem. 266(17):11341-11346, 1991; Greenberg et al., Proc. Natl. Acad. Sci. 90(24):12035-12039, 1993) for adipocytes; Ly-6C and Mac-1 (McCormack et al., J. Immunol. 151:6389-6398, 1993; Gordon et al., Current Opin. in Immunol. 4(25):25-32, 1992) and non-specific esterase (NSE; Yam et al., Amer. J. Clin. Path. 55:283, 1971) for cells of the monocytic lineage that includes macrophages and osteoclast precursors; and albumin, liver-specific glucokinase, liver-specific pyruvate kinase and the liver isozyme of glycogen synthase (Miller et al., J. Biol. Chem. 261:785-790, 1986; Magnuson, Diabetes 39: 523-527, 1990) for hepatocytes.

Progenitor or precursor cells can be stimulated to differentiate, thereby providing differentiated cells. Differentiation is induced by exposing the undifferentiated stem cells or precursor cells to factors that can be supplied by surrounding cells (paracrine regulation), by the cell itself (autocrine regulation) or by distant hormone-secreting cells (endocrine regulation). In addition, it has been demonstrated that certain factors can be added exogenously and will stimulate differentiation of cells. For example, osteoclasts are stimulated to differentiate by exposure to vitamin D and dexamethasone. Osteoblasts are induced to differentiate by exposure to retinoic acid, TGF-b or bone morphogenic proteins (BMP).

The present invention discloses that ob protein is a potent stimulator that drives osteogenic cells to become activated osteoblasts. Ob protein has been previously implicated as a regulator of appetite (Friedman

et al., WO 96/05309) and fertility (Chehab et al., Nature Genetics 12:318-320, 1995), but was previously not known to be a bone-stimulating factor. The receptor for ob protein has been reported to be found in brain, kidney and lung, but not in bone (Tartaglia et al., Cell 83:1263-1271, 1995).

When ob/ob mice are treated with leptin, a variety of effects are observed in cells of the mesenchymal lineage (which includes stem cells, stromal cells, osteoblast precursor cells and mature osteoblasts). A stimulation of proliferation and/or differentiation of mesenchymal lineage cells results in an increased number of mature osteoblasts, as determined by standard assays. Typically, test samples containing cells of the mesenchymal lineage are incubated in the presence of ob protein and in the absence of ob protein (control sample). The test samples are then scored for cell proliferation and differentiation by visual examination and/or staining. Such test samples may be *in vitro* cultured cells or may be obtained from an *in vivo* model system. In addition, exposure to ob protein can increase the size of the resultant mature osteoblasts. Alternatively, ob protein may stimulate the growth of isolated osteoblasts, as determined by [^{3}H]thymidine incorporation or [^{35}S]-labeling of proteins. Furthermore, exposure of mesenchymal lineage cells to ob protein may modulate the biological activity of the cells (such as up-regulation or activation of bone-stimulatory molecules), such that bone formation (i.e., *in vivo*) or a bone-forming molecule (i.e., *in vitro*) is induced. Particularly in an *in vivo* environment, ob protein may enhance recruitment or migration of bone-forming cells to the proper bone-forming tissues and tissue sites.

The effect of ob protein on bone formation or osteoblast activation may be either direct or indirect. It has been reported that there is an inverse relationship between the differentiation of adipocytes and osteogenic

cells *in vitro* (Beresford et al., J. Cell Sci. 102:341-351, 1992), and correlations between increased volume of marrow adipose tissue and osteoporosis have been established (Meunier et al., Clin. Orthop. Rel. Res. 80:147-154, 1971; Burkhardt et al., Bone 8: 157-164, 1987 and Minaire et al., Calcif. Tiss. Int. 36:338-340, 1984). These observations, in combination with the present invention, suggest the possibility that ob protein plays a role in the mesenchymal cell differentiation pathway, driving a common adipocyte-osteoblast progenitor to the osteogenic phenotype.

Bone marrow adipocytes are known to secrete factors that are required for the maturation of other cell types found in the bone marrow (Gimble, The New Biologist 2:304-312, 1990). Therefore, changes in levels of growth and differentiation factors due to shifts within the cell population in the bone marrow microenvironment would be expected to alter the osteoblast maturation process.

The effects of ob protein are believed to be both central and peripheral (Yu et al., Proc. Natl. Acad. Sci. USA 94:1023-1028, 1997). Thus, endocrine or CNS tissues or cell types that have been exposed to ob protein and then cultured may contain additional factors that contribute to the osteogenic effects. In particular, culturing of CNS cells, such as pituitary or hypothalamic cells, after exposure to ob protein may enhance the osteogenic effects.

PTH provides a paradigm for bone forming agents. At specific dose levels in ovariectomized mice, PTH increases osteoblast number and size (Lui et al., J. Bone Mineral Res. 5:973-981, 1990). This increase in osteoblast size and number corresponds with increases in cancellous bone formation.

Estrogen is an anti-resorptive agent and is used for treatment of osteoporosis. Adipocytes have been shown to produce estrogen (Heiss et al., J. Clin. Endocrinol. Metabolism 80:1591-1596, 1995), and may be a primary

source of estrogen in post-menopausal women. Low body fat level is considered a risk factor for osteoporosis. However, marrow fat has been shown to be increased in a variety of osteoporotic patients. When the bone loss stopped, the marrow fat levels decreased (Martin et al., Bone 12:123-131, 1991). Therefore, the relationship between peripheral adipose cells, marrow adipose cells, and bone formation and loss is complex and requires further elucidation.

In one embodiment of the present invention, a composition comprising ob protein is used as a therapeutic agent to enhance osteoblast-mediated bone formation. The methods of the invention may be applied to promote the repair of bone defects and deficiencies, such as those occuring in closed, open and non-union fractures; to promote bone healing in plastic surgery; to stimulate bone ingrowth into non-cemented prosthetic joints and dental implants; in the treatment of peridontal disease and defects; to increase bone formation during distraction osteogenesis; and in treatment of other skeletal disorders that may be treated by stimulation of osteoblastic activity, such as osteoporosis and arthritis. *De novo* bone formation provided by the methods of the present invention would have use in repair of congenital, trauma-induced, oncologic resection of bone or healing bone following radiation-induced osteonecrosis (Hart et al, Cancer 37:2580-2585, 1976). The methods of the present invention may also find use in plastic surgery.

Growth hormone has been shown to decrease (and perhaps eliminate) some of the pathologic phenotypes seen in growing bone in the presence of excess glucocorticoids (Altman et al, Calcif. Tissue Int. 51:298-304, 1992). Ob polypeptides have been shown to augment growth hormone releasing hormone (GHRH) stimulation of growth hormone from cultured pituitary cells. Many of the peripheral actions of growth hormone, including *de novo* bone formation, are mediated by IGF-I. The methods of the

present invention result in increased levels of IGF-I, consistant with a putative pathway in which ob protein exerts an effect on bone.

The compositions of the present invention may be administered systemically or locally. For systemic use, ob proteins are formulated for parenteral (e.g., intravenous, subcutaneous, intramuscular, intraperitoneal, intranasal or transdermal) or enteral (e.g., oral or rectal) delivery according to conventional methods. Intravenous administration will be by a series of injections or by continuous infusion over an extended period. Administration by injection or other routes of discretely spaced administration will generally be performed at intervals ranging from weekly to once to three times daily. Treatment will continue until the desired outcome is achieved. In general, pharmaceutical formulations will include an ob protein in combination with a pharmaceutically acceptable vehicle, such as saline, buffered saline, 5% dextrose in water, borate-buffered saline containing trace metals or the like. Formulations may further include one or more excipients, preservatives, solubilizers, buffering agents, albumin to prevent protein loss on vial surfaces, lubricants, fillers, stabilizers (for example, soluble receptors), etc. Methods of formulation are well known in the art and are disclosed, for example, in Remington's Pharmaceutical Sciences, Gennaro, ed., Mack Publishing Co., Easton PA, 1990, which is incorporated herein by reference. Pharmaceutical compositions for use within the present invention can be in the form of sterile, non-pyrogenic liquid solutions or suspensions, coated capsules, suppositories, lyophilized powders, transdermal patches or other forms known in the art. Local administration may be by injection at the site of injury or defect or by insertion or attachment of a solid carrier at the site, or by direct, topical application of a viscous liquid.

Delivery of ob protein to wound sites may be enhanced by the use of controlled-release compositions such as those described in pending U.S. Patent Application No. 07/871,246 (corresponding to WIPO publication WO 93/20859 which is incorporated herein by reference in its entirety). Films of this type are particularly useful as coatings for prosthetic devices and surgical implants. The films may, for example, be wrapped around the outer surfaces of surgical screws, rods, pins, plates and the like. Implantable devices of this type are routinely used in orthopedic surgery. The films can also be used to coat bone filling materials, such as hydroxyapatite blocks, demineralized bone matrix plugs, collagen matrices and the like. In general, a film or device as described herein is applied to the bone at the fracture site. Application is generally by implantation into the bone or attachment to the surface using standard surgical procedures.

In addition to the copolymers and carriers noted above, the biodegradable films and matrices may include other active or inert components. Of particular interest are those agents that promote tissue growth or infiltration. Agents that promote bone growth or inhibit bone resorption, such as bone morphogenic proteins (U.S. Patent No. 4,761,471; PCT Publication WO 90/11366), osteogenin (Sampath et al., Proc. Natl. Acad. Sci. USA 84: 7109-7113, 1987) and NaF (Tencer et al., J. Biomed. Mat. Res. 23: 571-589, 1989) are preferred.

Alternative methods for delivery of ob protein include use of ALZET osmotic minipumps (Alza Corp. Palo Alto, CA); sustained release matrix materials such as those disclosed in Wang et al. (WO 90/11366); electrically charged dextran beads as disclosed in Bao et al. (WO 92/03125); collagen-based delivery systems, for example, as disclosed in Ksander et al. (Ann. Surg. 211(3):288-294, 1990); methylcellulose gel systems as disclosed in Beck et al. (J. Bone Min. Res. 6(11):1257-1265, 1991) and alginate-based systems as disclosed in Edelman et al.

(Biomaterials, 12:619-626, 1991). Other methods well known in the art for sustained local delivery in bone include porous coated metal protheses that can be impregnated, solid plastic rods with therapeutic compositions incorporated within them and porous hydroxyapatite/tricalcium phosphate ceramic as a carrier of growth factors (Toriumi et al., Laryngoscope 101:395-404, 1991).

Delivery of systemically administered compositions of the present invention may be enhanced by conjugating ob protein to a targeting molecule. "Targeting molecule" refers to a molecule that binds to the tissue of interest. For example, bone-targeting molecules include tetracyclines; calcein; bisphosphonates; polyaspartic acid; polyglutamic acid; aminophosphosugars; peptides known to be associated with the mineral phase of bone such as osteonectin, bone sialoprotein and osteopontin; bone specific antibodies; proteins with bone mineral binding domains and the like. See, for example, the disclosures of Bentz et al. (EP 0512844) and Murakami et al. (EP 0341961).

The methods of the present invention provide the use of ob protein for treating bone loss in conjunction with HRT and other antiresorptive agents, such as bisphosphonates and calcitonin.

Within the present invention, an "effective amount" of a composition is that amount which produces a statistically significant effect. When ob protein is used to stimulate the growth of osteoblast cells *in vitro*, it is generally desirable to produce an increase in growth of at least 50%, as measured by incorporation of ^{3}H-thymidine, as compared to cells grown the absence of ob protein. For therapeutic uses, an "effective amount" is the amount of the composition comprising ob protein required to provide a clinically significant increase in healing rates in fracture repair, reversal of bone loss in osteoporosis, stimulation and/or augmentation of bone

formation in fracture non-unions and distraction osteogenesis, increase and/or acceleration of bone growth into prosthetic devices and repair of dental defects. Such effective amounts will depend, in part, on the particular condition to be treated and other factors evident to those skilled in the art. For example in osteoporosis, increase in bone formation is manifested as a statistically significant difference in bone mass between treatment and control groups. This difference in bone mass may be seen, for example, as a 5-20% or more increase in bone mass in the treatment group. Other measurements of clinically significant increases in healing may include, for example, tests for breaking strength and tension, breaking strength and torsion, 4-point bending, increased connectivity in bone biopsies and other biomechanical tests well known to those skilled in the art. General guidance for treatment regimens is obtained from experiments carried out in animal models of the disease of interest.

In vitro, the preferred range for ob protein concentration is about 1 pg/ml to 100 ng/ml, preferably 50 pg/ml to 5 ng/ml.

Therapeutic doses will generally be in the range of 10 µg/kg to 100 mg/kg of patient weight per day, preferably 0.1-10 mg/kg per day, with the exact dose determined by the clinician according to accepted standards, taking into account the nature and severity of the condition to be treated, patient traits, etc. Determination of dose is within the level of ordinary skill in the art. In general, sustained release compositions will be formulated to provide doses in the higher ends of the stated ranges. Doses will be adjusted to the release rate. Initial dose determinations can be made by extrapolation from animal models according to standard principles, taking into consideration route of administration and pharmacokinetic factors such as rate of absorption, distribution, biotransformation,

bioavailability and rate of excretion. See, for example, Goodman and Gilman, eds., The Pharmacological Basis of Therapeutics, Fifth Edition, MacMillan Publishing Co., Inc., New York, 1975.

The compositions described above are administered over a period ranging from a day to 6 months or more, depending on the condition to be treated. In general, doses will be adminstered from 5 times a day to once a month, and preferably from once a day to once a month until healing is substantially complete or bone mass increase is achieved. The actual treatment regimen will depend upon such factors as the age and general condition of the patient, the condition to be treated, and the route of delivery. Determination of treatment regimen is within the level of ordinary skill in the art.

Examples

Example 1. Analysis of Mouse Tibias

A. Female Mice

Ten-week-old C57BL/6J *ob/ob* female mice (Jackson Labs, Bar Harbor, ME) were treated with vehicle or ob protein (50 μg twice per day i.p., total 100 μg/day/animal) for 14 days. All mice were pair-fed throughout the experiment. To achieve matched food intake between the ob-treated and vehicle-treated animals, the pairs were initially matched for age and body weight. The pair mate for ob treatment was injected a fed and 24 hours later vehicle-treated animals were injected and food intake was restricted to the amount consumed by the ob-treated pair mate. After 14 days, the animals were sacrificed and the tibias were removed. Tibial bone samples were fixed in 10% neutral buffered formalin, decalcified in 5% formic acid with 10% sodium citrate, washed in tap water, dehydrated in a series of 70%-100% ethanol, and embedded in glycol methacrylate. The

proximal end of the tibia (about 5 mm long) was cut frontally at 5 μm, stained for tartrate-resistant acid phosphatase (TRAP) activity, and counter-stained with methyl green and thionin for identification of bone cells.

Osteoblasts were identified by central negative (clear) Golgi area, eccentric nucleus, and the strong basophilic counter-stain of methyl green and thionin, while osteoclasts by TRAP stain, multinucleation, and non-uniform shape. The following bone parameters were evaluated for histomorphometric changes.

1) Growth plate activity: width measured every 50-100 μm at 42 X magnification to determine the growth plate activity.

2) Number of endocortical osteoblasts: measured along one side of endocortical surface at 212 X magnification.

3) Endocortical osteoblast size: measured using all the osteoblasts counted in the vehicle-treated mice or 50 osteoblasts randomly selected in the ob-treated mice at 424 X magnification.

4) Number of endosteal osteoblasts: measured along the endosteal surface of cancellous bone in the metaphysis at 212 X magnification in a zone area 0.62-3.10 mm distal to the growth plate.

5) Number of endosteal osteoclasts: measured simultaneously when endosteal osteoblast counts were taken.

6) Percentage of cancellous bone (bone volume/tissue volume, BV/TV): calculated from cancellous bone area per referent tissue area, and measured in the same reference areas where endosteal osteoblast and osteoclast counts were taken.

7) Number of epiphyseal osteoblasts: measured from endosteal surface of cancellous bone in the epiphysis to determine if osteoblasts were also activated in this metabolically inactive bone site.

Analysis of the data (Mean ± SEM, n=5/group) demonstrated the following:

1. Ob protein treatment significantly increased growth plate activity as measured by increased growth plate width.

2. Ob protein treatment significantly increased the number of osteoblasts at all the bone sites examined, including endocortical surface and endosteal surfaces in the metaphysis and epiphysis, with the most dramatic change occurring at the endocortical surface, which was about 32-fold greater than the level in the control group. Moreover, the size of osteoblasts increased more than 2-fold above the control value. These results suggest that ob protein stimulated osteoblast activity.

3. Ob protein treatment did not significantly change the number of endosteal osteoclasts, suggesting that bone resorption levels were not altered.

4. Percentage of cancellous bone volume was not significantly changed by ob protein, suggesting that the bone-forming effects of ob protein are sequential and may require a longer time to see mineralized bone, as similar experiments using PTH have shown.

B. Male Mice

An identical experiment performed using C57BL/6J *ob/ob* male mice provided similar results, suggesting that the mechanism underlying the observed osteoblast activation and bone morphology changes is not estrogen-mediated.

Example 2. Bone Mineralization

Osteoblast-mediated bone mineralization following exposure of osteoblasts to ob protein was analyzed using the following *in vitro* assay:

24-well plates (American Scientific Products, Chicago, IL) were coated with rat tail collagen type 1 (Collaborative Research, Inc., Bedford, MA) by mixing 300

ul of 1 mg/ml rat tail collagen with 14.7 ml of 0.02 N acetic acid. The mixture was added at 2 ml/well and incubated for 1 hr at room temperature.

After incubation the wells were rinsed with 2.5 ml PBS twice, and set in a hood to dry for 1 hr. Once dry, the plates were stored at 4°C.

Osteoblast cells were plated so as to be 90-100% confluent by the next day. The cells used were derived from p53 knockout mice (see WO 96/07733) and plated at approximately 5 x 10^4 cells/well for cell lines 2-45 and CCC-4, and at 1 x 10^5 cells/well for cell line 2-29 using growth medium (Table 1) at 1-2 ml/well and vehicle or serial dilutions of ob protein that ranged 50 ng/ml to 5 pg/ml.

Table 1

Growth Medium

alpha-MEM (JRH Biosciences, Lenexa, KS)
15% fetal calf serum (Hyclone, Logan, UT)
(1mM) sodium pyruvate (Irvine, Santa Ana, CA)
(0.29 mg/ml) L-glutamine (Hazelton, Lenexa, KS)
1x PSN (5 mg/ml penicillin, 5 mg/ml streptomycin, 10 mg/ml neomycin) (Gibco BRL, Gaithersburg, MD)

Beta-Glycerophosphate (Sigma, St. Louis, MO) was added to a final concentration of from 2 to 10 mM. L-Ascorbic acid phosphate magnesium salt *n*-hydrate (Wako Pure Chemical Industries, Ltd., Osaka, Japan) was added to final concentration of 50 ug/ml.

The cells were fed every 2-4 days with medium containing beta-glycerophosphate, L-ascorbic acid and ob protein or vehicle.

Prior to measurement of mineralization, the wells were rinsed with PBS, and the mineralized layer was dissolved by adding 300 ul/well of 0.5 N HCl and incubating overnight at room temperature.

After incubation, the solution was buffered by mixing 250 ul of the 0.5 N HCl dissolved mineralized layer and adding 600 ul 2 M Tris, pH 7.4, 600 ul Nova 7/9/10 urine diluent (Nova Biomedical, Waltham, MA) and 50 ul 2N-NaOH. Total calcium was measured using the Nova 7/7+7 Electrolyte analyzer (Nova Biomedical).

Results demonstrated that during days 1-6, mineralization was increased for cells treated with 500 pg/ml over vehicle-treated cells.

Example 3. Calvarial Assay

The effects of ob protein on bone growth were tested in ten-week-old CD-1 male mice (Jackson Labs) by injecting ob protein into the subcutaneous tissue over the calvarium of the mice. Doses ranged from 0.001-5.0 mg/mouse, given three times daily for five days.

After 14 days, the mice were sacrificed and calvarial bone growth was measured by histomorphometry. Parameters measured are as described in Example 1. Results were negative, with no significant mineralization seen in the calvaria.

Example 4. Ovariectomized Rat Assay

The ovariectomized rat is accepted as an animal model of human post-menopausal osteoporosis. To assess the effects of systemic administration of ob protein on skeletal tissues in an animal model of acute bone loss related to estrogen deficiency similar to that seen in post-menopausal women, female normal rats are either sham-operated or surgically ovariectomized. Seven days after surgery, treatment is begun, administering either vehicle, ob protein or estrogen (160 µg/kg, subcutaneously). Prior to sacrificing the animals, single doses of tetracycline or demeclocycline are administered to assess bone formation and mineralization. Tibias and lumbar vertebrae are removed, fixed, processed and analyzed as described in Example 1.

Example 5. Histomorphometric Examination of Leptin Treated ob/ob mice

Thirteen-week-old genetically obese mice (ob/ob, C57BL/6J) were weight-matched, divided into 5 groups and treated as follows:

PBS 28 days (n=4)
Vehicle 14 days (14 d on and 14 d off) (n=6)
Leptin 14 days (14 d on and 14 d off) (n=7)
Vehicle 28 days (n=6)
Leptin 28 days (n=6)

Both vehicle (0.007 mM borate in PBS) and leptin were given twice daily by i.p. injections (leptin 50 μg 2X/day, total 100 μg). Mice were given food and water ad libitum. Calcein injections (15 mg/kg body weight) were given 9 and 2 days before sacrifice to label the newly formed bone for assessment of dynamic bone changes. All animals were sacrificed at the end of day 28. Tibial bone samples were fixed in 70% ethanol and embedded in methyl methacrylate without decalcification. The proximal end of the tibia (about 5 mm long) was cut parasagittally at 5 and 10 μm. Ten-μm sections were mounted without staining for evaluation of calcein labels, while 5-μm sections were stained for tartrate-resistant acid phosphatase (TRAP) activity and counter-stained with methyl green and thionin for identification of bone cells. Osteoblasts were identified by central negative (clear) Golgi area, eccentric nucleus, and the strong basophilic stain, while osteoclasts by TRAP stain, multinucleation, and non-uniform shape.

The following bone sites were evaluated for histomorphometric changes:

A. Endocortical bone

Endocortical bone was evaluated at both the anterior and the posterior region within 3.7 mm below the

growth plate at 90X magnification. In general, bone-forming activity can differ significantly at various sites within the bone, with greater activity at the posterior than the anterior region of the bone site evaluated.

The evaluations were as follows:

1) percent single labeled surface (sLS): the percentage of bone surfaces with single calcein labels.

2) percent double labeled surface (dLS): the percentage of bone surface with double calcein labels.

3) mineral apposition rate (MAR, μm/d): the width of mineralized bone deposited per day on the endocortical bone surface.

4) bone formation rate (BFR/BS, μm3/μm2/day): The amount of new bone formed per unit bone surface per day (bone surface referent).

B. Metaphysis

Because changes in bone cell activities following leptin treatment could differ in the cancellous bone directly below (primary spongiosa) and further away from the growth plate (secondary spongiosa), histomorphometric bone changes were evaluated at both sites.

The following dynamic bone changes were evaluated in the secondary spongiosa at 180X magnification and 0.62 mm distal to the growth plate:

1) percent single-labeled surface
2) percent double-labeled surface
3) mineral apposition rate
4) bone formation rate(BFR/BS)
5) number of endosteal osteoblasts (#osteoblasts/mm cancellous bone perimeter)
6) number of endosteal osteoclasts (#osteoclasts/mm cancellous bone perimeter)

7) percent cancellous bone volume (bone volume/tissue volume, BV/TV)

In the primary spongiosa, only the last three bone parameters were evaluated at 360X magnification and within 0.3 mm from the growth plate.

C. Epiphysis

At this bone site, bone cells are usually less responsive compared with the other two bone sites (e.g., do not respond to short-term estrogen deficiency or treatment). Osteoblasts, however, were active following leptin treatment in ob/ob mice as shown previously. To confirm this unusual finding, dynamic bone changes were further evaluated. The following bone parameters were evaluated from the entire endosteal surface of the cancellous bone at 180X magnification:

1) percent single-labeled surface
2) percent double-labeled surface
3) mineral apposition rate (MAR, μm/day)
4) bone formation rate (BFR/BS, μm3/μm2/day)

Data (Mean ± SD) obtained from this study are summarized as follows:

1. Leptin treatment of ob/ob mice significantly increased the tibial endocortical bone formation at both the anterior and the posterior sites as indicated by increased % single- and/or double-labeled surfaces, mineral apposition rate, and bone formation rate, confirming the earlier findings of increased osteoblast activity by leptin treatment.

2. 14 days of leptin treatment only partially retained this increased endocortical bone forming activity.

3. Similar stimulation of bone formation by leptin was found in the tibial metaphysis as indicated by dynamic indices of bone formation (secondary spongiosa), but it was less dramatic (e.g., the number of endosteal osteoblasts was increased following 28 days of leptin

treatment, but the increase was not statistically significant, although it was significant when evaluated in the primary spongiosa).

4. In contrast to the earlier leptin experiment, the number of endosteal osteoclasts in the secondary spongiosa was significantly increased by letpin. This increase, however, was not significant in the primary spongiosa.

5. As previously observed, the % cancellous bone volume was not significantly changed by the leptin treatment.

6. In the epiphysis, leptin treatment resulted in significant increases in % single-labeled surface, % double labeled surface and bone formation rate.

7. The stimulation of bone formation by leptin parallels with the increased serum IGF-I levels following leptin treatment and may indicate its potential role in mediating leptin effects on osteoblasts in ob/ob mice.

8. Present results suggest that bone defects associated with glucocorticoid excess (Dubuc et al. Horm. Metab. Res. 7:102, 1975 and Bray et al., Physiological Rev. 59:719-809, 1979) and/or diabetes could be normalized or ameliorated by leptin treatment.

9. A combined treatment with an anti-resorptive agent could potentially augment the anabolic effect of leptin on bone.

From the foregoing, it will be appreciated that, although specific embodiments of the invention have been described herein for purposes of illustration, various modifications may be made without deviating from the spirit and scope of the invention. Accordingly, the invention is not limited except as by the appended claims.

SEQUENCE LISTING

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(ii) TITLE OF INVENTION: METHODS FOR INDUCING BONE FORMATION

(iii) NUMBER OF SEQUENCES: 4

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(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.25

(vi) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER:
(B) FILING DATE:
(C) CLASSIFICATION:

(viii) ATTORNEY/AGENT INFORMATION:
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(C) REFERENCE/DOCKET NUMBER: 96-07PC

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(B) TELEFAX: 206-442-6678

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 966 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 46..546

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

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(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 167 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

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35 40 45

Gln Ser Val Ser Ser Lys Gln Lys Val Thr Gly Leu Asp Phe Ile Pro
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Gly Leu His Pro Ile Leu Thr Leu Ser Lys Met Asp Gln Thr Leu Ala
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Leu Asp Leu Ser Pro Gly Cys
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(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 504 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 1..501

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

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            20                  25                  30

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65                  70                  75                  80

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Glu Ser Leu Asp Gly Val Leu Glu Ala Ser Leu Tyr Ser Thr Glu Val
    130                 135                 140

GTG GCT TTG AGC AGG CTG CAG GGC TCT CTG CAG GAC ATT CTT CAA CAG      480
Val Ala Leu Ser Arg Leu Gln Gly Ser Leu Gln Asp Ile Leu Gln Gln
145                 150                 155                 160

TTG GAT GTT AGC CCT GAA TGC TGA                                     504
Leu Asp Val Ser Pro Glu Cys
                165
```

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 167 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

```
Met Cys Trp Arg Pro Leu Cys Arg Phe Leu Trp Leu Trp Ser Tyr Leu
 1               5                  10                  15

Ser Tyr Val Gln Ala Val Pro Ile Gln Lys Val Gln Asp Asp Thr Lys
            20                  25                  30

Thr Leu Ile Lys Thr Ile Val Thr Arg Ile Asn Asp Ile Ser His Thr
        35                  40                  45

Gln Ser Val Ser Ala Lys Gln Arg Val Thr Gly Leu Asp Phe Ile Pro
    50                  55                  60

Gly Leu His Pro Ile Leu Ser Leu Ser Lys Met Asp Gln Thr Leu Ala
65                  70                  75                  80

Val Tyr Gln Gln Val Leu Thr Ser Leu Pro Ser Gln Asn Val Leu Gln
                85                  90                  95
```

Ile Ala Asn Asp Leu Glu Asn Leu Arg Asp Leu Leu His Leu Leu Ala
100 105 110

Phe Ser Lys Ser Cys Ser Leu Pro Gln Thr Ser Gly Leu Gln Lys Pro
115 120 125

Glu Ser Leu Asp Gly Val Leu Glu Ala Ser Leu Tyr Ser Thr Glu Val
130 135 140

Val Ala Leu Ser Arg Leu Gln Gly Ser Leu Gln Asp Ile Leu Gln Gln
145 150 155 160

Leu Asp Val Ser Pro Glu Cys
165

CLAIMS

We claim:

1. A method for stimulating a cell population containing marrow mesenchymal cells comprising:

exposing the cell population to an amount of ob protein effective for expansion of osteogenic cells.

2. The method of Claim 1 wherein the cell population is enriched for marrow mesenchymal cells.

3. The method of Claim 1 wherein hematopoietic cells have been removed from the cell population.

4. The method of Claim 1 wherein the osteogenic cells are predominantly osteoblasts.

5. The method of Claim 1 further comprising, after the step of exposing, the step of combining the cell population with an anti-resorptive agent.

6. The method of Claim 1 further comprising, after the step of exposing, the step of infusing the cell population into a mammal.

7. A method for stimulating a cell population containing marrow mesenchymal cells comprising:

exposing the cell population to a biological fluid obtained from an ob protein-treated mammal.

8. The method of Claim 7 wherein the biological fluid is serum.

9. The method of Claim 7 wherein the biological fluid has been fractionated to enrich for molecules effective for expansion of osteogenic cells.

10. A method for stimulating a cell population containing marrow mesenchymal cells comprising:

exposing the cell population to culture medium that has been conditioned by growth of endocrine or CNS cells or tissue exposed to ob protein.

11. The method of Claim 10 wherein the cells are pituitary or hypothalamic cells.

12. A method for promoting bone repair or bone healing in a mammal comprising:

administering to the mammal an amount of ob protein effective to provide a clinically significant increase in bone repair or bone healing rate.

13. A method for stimulating bone ingrowth into a prosthetic device or dental implant that has been inserted into a mammal, comprising:

administering to the mammal an amount of ob protein effective to provide a clinically significant increase in bone growth into the device or implant.

14. A method for treating bone loss in a mammal comprising:

administering to the mammal an amount of ob protein effective to provide a clinically significant increase in bone mass.

15. A method for increasing bone length in a mammal comprising:

administering to the mammal an amount of ob protein effective to provide a clinically significant increase in bone growth plate width.

16. The method of Claim 15 wherein the mammal is an adolescent or pre-adolescent.

17. A method for stimulating active bone growth in a mammal having a fracture in a bone growth plate, comprising:

administering to the mammal an amount of ob protein effective to restore a pre-fracture bone length.

18. A method for inducing bone formation in a mammal having an oncological resection of a bone, comprising:

administering to the mammal an amount of ob protein effective to induce bone formation at the site of resection.

INTERNATIONAL SEARCH REPORT

Intern al Application No
PCT/US 97/06892

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 A61K38/22 C12N5/06 C12N5/08

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 A61K C12N C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	GB 2 292 382 A (ROCKEFELLER UNIVERSITY) 21 February 1996 see claims 61-63,71,72,76 & WO 96 05309 A cited in the application	1-18
A	THE JOURNAL OF CLINICAL INVESTIGATION, vol. 96, October 1995, NEW YORK, N.Y., US, pages 2065-2070, XP000601377 D.S. WEIGLE ET AL.: "RECOMBINANT ob PROTEIN REDUCES FEEDING AND BODY WEIGHT IN THE ob/ob MOUSE." see the whole document	1-18
E	WO 97 18833 A (AMGEN INC.) 29 May 1997 see page 5, line 25 - line 28; claims 1,4; example 6 see page 25, line 1 - line 8	14

☐ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

1

Date of the actual completion of the international search	Date of mailing of the international search report
8 August 1997	26.08.97

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax (+31-70) 340-3016	Ryckebosch, A

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 97/06892

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.: 6,12-18
because they relate to subject matter not required to be searched by this Authority, namely:

Remark: Although claim(s) 6,12-18 is(are) directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

2. [ ] Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

information on patent family members

Inter nal Application No
PCT/US 97/06892

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
GB 2292382 A	21-02-96	AU 3329895 A	07-03-96
		CA 2195955 A	22-02-96
		DE 19531931 A	07-03-96
		EP 0777732 A	11-06-97
		FI 970656 A	17-02-97
		NO 970683 A	16-04-97
		PL 319021 A	21-07-97
		WO 9605309 A	22-02-96
		ZA 9506868 A	09-04-96
		JP 9502729 T	18-03-97
WO 9718833 A	29-05-97	NONE	